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ABSTRACT

Tumor progression is characterized by altered cell-cell interactions, increased invasion and angiogenesis, and upregulation of integrins including α5 and αv; as well as increased tenascin (TN) and fibronectin (FN) deposition. We asked whether elevated α5 integrin could promote malignant progression by compromising tissue polarity and promoting angiogenesis. Using the HMT3522 breast cancer progression cell series and 3D reconstituted basement membrane (rBM) co-culture and xenograft assays we found that malignant transformation correlated with loss of tissue polarity, acquisition of invasiveness, increased α5 integrin expression, VEGF and IL-8 upregulation, and a pro-angiogenic phenotype in culture and in vivo. However, only inhibiting α5β1 activity could phenotypically revert these tumors, reduce invasion and impair angiogenesis in culture and in vivo. Moreover, overexpression of α5 integrin in S-1 nonmalignant cells compromised polarity and induced angiogenesis in vitro and in vivo. Thus, we propose that α5β1 integrin ligation of FN regulates tissue architecture- mediated (TAM)-dormancy through cooperative interaction with EGFR and induction of angiogenesis.

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2005 Progress report for Award number DAMD17-03-1-0421
Principal Investigator: **Rozenberg, G.I.**

Introduction:
Tumor dormancy is a condition in which tumor cells persist in the host for a long period of time but do not grow. It is observed in several solid tumor types, including breast cancer. Moreover, occult primary cancers are often found incidentally during autopsies of individuals who did not die of cancer, breast cancer being detected in 30% of autopsied women. Various factors have been identified as possible contributors to tumor dormancy and subsequent recurrence, including tumor angiogenesis (1-3), balance between cell proliferation and apoptosis, immune regulation (4) and cancer cell interactions with the microenvironment (5,6). The notion that cancer is a disease of altered tissue architecture is becoming more and more accepted. A resurgence of Dr Mintz's 1970s (7) idea about tissue context being a potent regulator of the malignant phenotype of cancer cells has been translated now into the "Tissue organization field theory of carcinogenesis" (5,6). This theory proposes that cancer development disrupts normal interactions between adjacent cells or stroma that dramatically modify the ability of cells to sense normal regulatory signals inherent in the structure of epithelial tissues.
Cells interact with the microenvironment mainly through integrins, which are alpha-beta heterodimers that recognize different ligands present in the extracellular matrix (ECM) and in the surface of neighboring cells. Integrins are key regulators of growth, survival, adhesion, invasion and metastasis, as well as of angiogenesis; in fact, different cancers often show altered integrin expression and alterations in ECM composition. Indeed, the reactive stroma found in breast tumors and other types of cancers is composed of high amounts of FN and TN (8-10). Our working hypothesis is that *increased expression of provisional ECM proteins such as FN and TN and the integrins α5, αv, β1 and β6 compromise mammary tissue organization to induce a proangiogenic and invasive malignant phenotype in mammary epithelial cells (MECs).* More specifically, the aim of our work was to underscore the mechanisms governing tissue architecture- mediated (TAM) tumor dormancy. We used a tractable mammary tumor progression model (HMT3522) in combination with 3D rBM assays and co-cultures of endothelial cells (ECs) and MECs as well as in vivo studies, to show that α5β1 integrin ligation of FN regulates TAM- tumor dormancy through cooperative interaction with epidermal growth factor receptor (EGFR) and induction of angiogenesis.

Body:
In my first year, I reported the phenotypic reversion potential of α5β1 integrin in the tumor cells, the preparation of nonmalignant cells expressing α5 integrin and the design of a new 3D MEC-EC co-culture model. Over the course of my second year I have worked with all of these in vitro and in vivo, to further study the tumorigenic and angiogenic properties of α5β1 integrin. With these studies I can report significant progress on all three of my specific aims and the tasks detailed in my SOW:

1) To determine if there is a correlation between tumorigenicity, loss of acinar structure, αv and/or α5 integrin expression and angiogenesis.
2) To test if the reacquisition of a polarized acinar structure is related to the loss of the angiogenic phenotype and if this is linked to changes in αv and/or α5 integrin.
3) To determine if the upregulation of αv and/or α5 integrins is a predictor of malignant behavior in HMT-3522 MECs, and functions by compromising tissue organizaztion and inducing angiogenesis and invasion.

I would like to point that I have altered the schedule of the specific tasks and the actual tasks in my SOW depending on the results obtained in preceding experiments.

Progression:

The in vitro studies using 3D rBM showed that while S1 nonmalignant cells form polarized acini that growth arrest and do not deposit FN or TN, tumor T4-2 cells lose polarization, are still growing (positive Ki-67 staining) and are invasive, and deposit high amounts of FN and TN. Phenotypic reversion of the tumors leads to re-polarization of the structures and repression of FN and TN deposition, together with controlled growth (Fig 1A). In vivo studies using S1 control and S1 α5 integrin expressing cells, as well as T4-2 cells with or without pre-incubation with α5 integrin function-blocking antibody (Ab) (Tasks 1g, 2Bg and 3i) showed that α5β1 integrin is involved in the progression towards malignancy, and that this is correlated to an increased angiogenic phenotype, since control nonmalignat MECs formed predominantly acinar/ductal structures with high levels of apoptosis when injected into nude mice, whereas nonmalignant MECs with ligated α5β1 integrin gave rise to hyperplastic lesions with absence of apoptosis. Tumor cells that express high levels of α5 integrin formed big tumor masses that were highly proliferative with a low grade of apoptosis, whereas, consistent with the notion that α5β1 integrin regulates tissue architecture to modulate tumor dormancy, inhibition of α5 integrin led to impaired tumor growth and increased apoptosis, resulting in the formation of big cysts accompanied by small portions of tumor (Fig1B). Coincidently with modulation of TAM-tumor dormancy in vivo, we observed a significant angiogenic response in association with α5β1 integrin activity such that nonmalignant cells with activated α5 integrin induced a robust angiogenic phenotype, similar to that observed in control tumors. While inhibition of α5 integrin function in tumor cells completely inhibited angiogenesis (Fig1C). The angiogenic switch that regulates tumor dormancy is linked to the balance between pro and anti-angiogenic factors. Accordingly, we observed a correlation between α5 integrin expression, tissue polarity and secretion of proangiogenic factors (Tasks 2Ac, 2Bc and 3d).

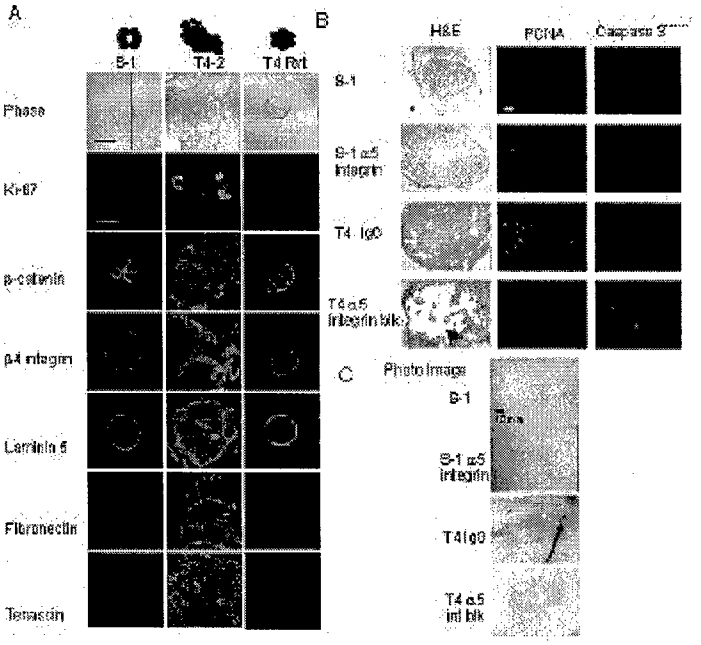

Fig 1: A) Micrographs and immunostaining of 3D rBM cultures of S1, T4-2 and T4-2 reverted cells showing different polarity markers and Ki-67 staining. B) Immunohistochemistry of in vivo outgrowths of S1, S-1 α5 integrin expressing, IgG-treated and S-1 control and α5 integrin function-blocking Ab-treated T4-2 cells. Column 1: H&E; 2: PCNA and 3: cleaved caspase 3 staining. Bar size: 100 μm. C) Photo image of the same outgrowths showing angiogenesis

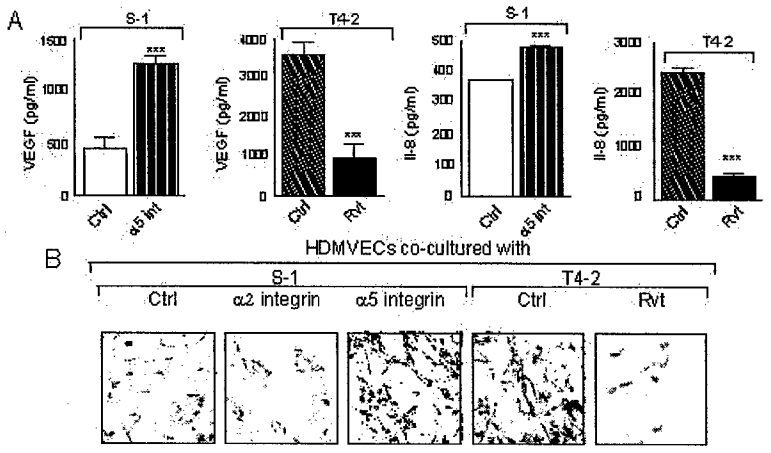

Fig 2: A) VEGF and Interleukin-8 levels present in conditioned media of S-1 control and S-1 α5 integrin expressing cells (left) and T4-2 and T4-2 revertants (right) grown in 3D rBM +/-FN.B) Micrographs of ECs co-cultured with S-1 control, S-1 α5 integrin and S-1 α2 integrin expressing cells and T4-2 and T4-2 revertants grown in 3D rBM +/-FN.

Tumor cells that express high levels of α5 integrin and induce a robust angiogenic phenotype in vivo expressed high levels of vascular endothelial growth factor (VEGF) and interleukin-8 (Il-8), but these were repressed by inhibition of α5 integrin and reformation of polarized acini, resulting in

loss of angiogenic behavior in 3D co-culture experiments (Tasks 2Ad, 2Be and 3g) (Fig 2).

Similarly, nonmalignant MECs with activated α5 integrin but not α2 integrin showed significant increase in VEGF and Il-8 secretion in concert with their enhanced angiogenic behavior in vitro (Fig 2).

Another important finding was that α5β1 integrin-FN interaction modulates tissue behavior by regulating PI3 and ERK kinases (Tasks 2Bc, 3b and 3d), since EGF stimulation of S1 α5 integrin-expressing or T4-2 cells was enough to elicit sustained stimulation of AKT and ERK activity, while S1 α5 integrin cells not expressing the transgene (+ tetracyclin) or T4-2 cells pre-incubated with α5 integrin function-blocking Ab could just elicit transient activation of these signaling molecules (Fig3A). Moreover, inhibition of PI3K or MEK induced phenotypic reversion of both S1 α5 integrin-expressing or T4-2 3D rBM cultures (Fig 3B).

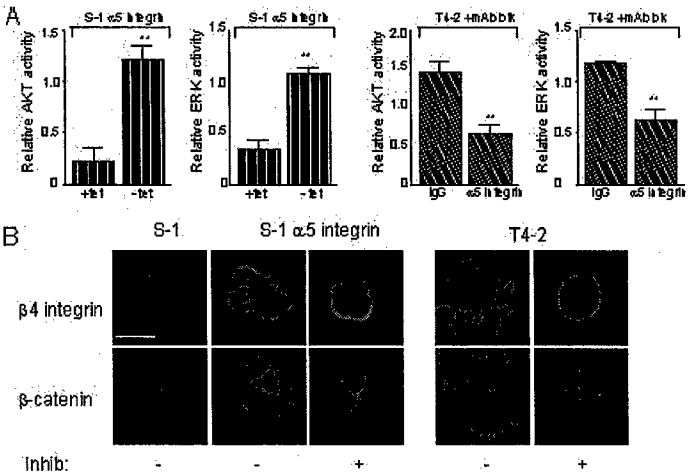

Fig 3: A) Graphs showing relative (phosphorilated over total) AKT and ERK activity in S-1 α5 integrin expressing or non expressing (+tet) cells and T4-2 cells incubated in the presence or absence of α5 integrin function-blocking Ab plated on FN, after 90 min of EGF stimulation. B) Immunostaining for different polarity markers of S-1 control and S-1 α5 integrin expressing cells grown in 3D rBM +FN without (-) or with (+) MEK, EGFR or PI3K inhibitor (left panel) and T4-2 cells grown in 3D rBM in the same conditions (right panel). Bar size: 20 μm.

Key Reserch Accomplishments:

- Transcriptional characterization (Microarray analysis) of S1, S3 and T4-2 cells is still in progress.
- I have prepared an α2 integrin retroviral inducible expression construct and retrovirus (not included in the approved SOW)
- I have successfully prepared and characterized a pooled population of S1 cells expressing α2 integrin, and done protein expression characterization by FACS analysis in 2D as well as immunological and protein expression characterization in 3D rBM (not included in the approved SOW).
- I have done protein expression characterization of S-1 α5 and α2 integrin-expressing and T4-2 reverted (including EGFR inhibition or β1 or α5 integrin blocking) cells in 3D rBM.
- I have done angiogenesis characterization of S-1 α5 and α2 integrin-expressing and T4-2 reverted cells in 3D co-cultures.
- I have done in vivo studies with S-1 control and S1 α5 integrin expressing cells, as well as T4-2 cells with or without pre-incubation with α5 integrin function-blocking Ab and assessed their tumorigenic and angiogenic potential.
- I have done studies to determine which signaling molecules are involved in the effects achieved by α5β1 integrin-FN ligation (not included in the approved SOW)

Reportable outcomes:

Research:

Abstracts:

-Gabriela I. Rozenberg, J. Friedland, J.N. Lakins, A.L. Sieminski, C. Mies, K. Gooch, J.C. Calvo and V.M. Weaver. "Tissue Polarity Regulates Angiogenesis and Tumor Dormancy Through α5β1 Integrin-EGFR-uPAR crosstalk". EMBO, 3rd IRCC International Cancer Conference; Candiolo, Turin, Italy. 2005

-Gabriela I. Rozenberg, J.N. Lakins, C. Chatterjee, J. Friedland and V.M. Weaver. "Integrin α5β1-fibronectin interactions and the metastatic phenotype".44th Annual Meeting of the American Society for Cell Biology; Washington, D.C., USA. 2004.

-Gabriela I. Rozenberg, J.N. Lakins, C. Chatterjee, J. Friedland and V.M. Weaver. "Integrin α5β1-fibronectin interactions and the metastatic phenotype". IME 2004 Symposium; Philadelphia, PA, USA. 2004. **Received Best Poster Award.**

Products:

-Prepared α2 integrin retroviral inducible expression constructs and retroviruses

-Prepared and characterized pooled populations of S1 cells expressing α2 integrin.

Conclusions:

With this and my previous work I have established that malignant transformation and breast cancer behavior is associated with α5β1 integrin overexpression, and that inhibition of this receptor can induce a state of tumor dormancy that is tightly linked to tissue architecture, that we named tissue architecture- mediated (TAM)- dormancy. This is supported by the fact that inhibition of α5β1 integrin activity in tumor cells in vivo was able to repress tumor growth, confirming previous results obtained in 3DrBM and soft agar assays with integrin function-blocking Abs. This was linked to inhibition of angiogenesis in vivo and in 3D co-cultures and correlated with downregulation of proangiogenic molecules such as VEGF and Il-8.

Moreover, ectopic expression of α5β1 integrin by the nonmalignant S1 cells induced loss of tissue structure (hyperplasia) in vivo, confirming previous results that showed loss of polarity in 3D rBM+FN by these cells. This was also correlated with an increased angiogenic phenotype in vivo and in 3D co-cultures, and with the upregulated production of VEGF and Il-8 by these cells compared to S1 control cells.

For my next year, I want to further study the molecules that regulate these processes. Since tumor dormancy has been also tightly linked to urokinase-type plasminogen activator receptor (uPAR) deficit, and uPAR levels increase in cancer (11). And moreover, it has been shown that α5β1 integrin-uPAR interaction through EGFR is required for tumor growth in vivo, and that interfering with any of these receptors can induce tumor dormancy in hepatocarcinoma cells (12), I would like to analyze the uPAR status in the HMT-3522 cell series and determine via function blocking studies if this receptor is involved in TAM-dormancy through regulation of angiogenesis. Other remaining tasks for the next years will be to work with the pre-malignant S3 cells expressing α5 integrin, to assess their tumorigenic and angiogenic properties in vitro and in vivo.

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